

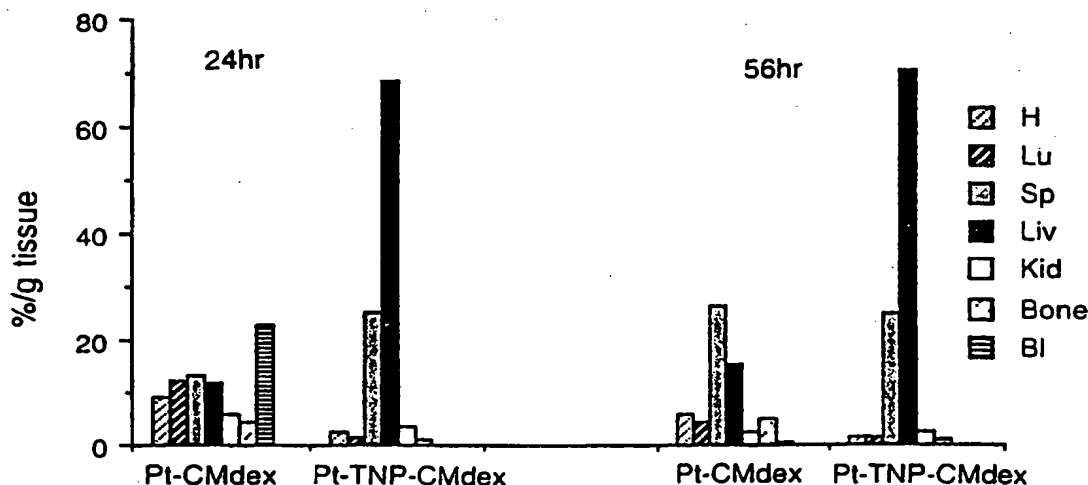
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(54) Title: X-RAY IMAGING OF TUMORS WITH DEXTRAN CARRIER OF PLATINUM COMPOUNDS



(57) Abstract

A delivery system and method for targeting a contrast agent to tissues and organs for improved diagnosis of tumors and for prognosis and follow-up of cancer therapy by X-ray imaging. The delivery system includes a conjugate or complex of a macromolecular carrier and a contrast agent selected from the group consisting of: (a) a compound having a heavy element with an atomic number in the range of 75 to 92, and (b) a compound having an element with a property of an abrupt change in its X-ray attenuation coefficient within the energy range used for radiography, said macromolecular carrier of said conjugate or complex being optionally linked to a specific tissue marker molecule.

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X-RAY IMAGING OF TUMORS WITH DEXTRAN CARRIER OF PLATINUM COMPOUNDS

5 FIELD OF THE INVENTION

The present invention relates to delivery systems for targeting contrast agents for improved diagnosis of tumors by X-ray imaging and to methods for diagnosis, prognosis and follow-up of treatment of tumors comprising
10 administration of the delivery systems to patients followed by X-ray imaging.

BACKGROUND OF THE INVENTION

Although treatment of breast carcinoma, the most
15 common malignancy among women, is improving steadily by means of surgical intervention and chemotherapy, prognosis depends heavily on early detection. In most developed countries, women aged 40-50 are urged to undergo annual mammography examinations. Ever since it was first introduced,
20 mammography has improved in terms of its spatial resolution and the decrease in the required radiation dose. Yet there are limits on the size of detectable lesions, within permissive radiation doses, that are dictated by the very small density differences between malignant and normal
25 tissues. Even the most advanced mammographic apparatus are limited in their tumor identification capacity. Although anatomical details are clearly portrayed, unequivocal identification is sometimes difficult.

The selective delivery of agents to specific organs
30 is intended in general to specifically localize a delivered agent in the target organ while maintaining low levels in blood and other organs. Ideally, a targeting vehicle, e.g., antibody, which binds selectively to epitopes on specific cells, may be used for targeted delivery of therapeutic or
35 diagnostic agents. Antibody-mediated delivery is, however, often restricted by lack of good antibody specificity (suitable antibodies for breast carcinoma are not available at present but they exist for other organs), limited specific uptake by the target tissue, low penetration and high

prolonged levels in blood and other organs (Epenetos et al, 1986; Welch, 1987).

Various other strategies have been developed for drug targeting, some of which are based on carrier-mediated delivery which employ natural ligands recognized by receptors on target cells. Most of these systems utilize macromolecular carriers armed with targeting ligands recognized by specific cell types. Certain targeting ligands are known, the most common are terminal saccharide residues recognized by receptors on liver parenchymal (Gal and GalNac of asialoglycoproteins, Ashwell et al, 1982) and non-parenchymal cells (GluNac and Man, Taylor et al, 1992), B-cells (Lasky et al, 1989) or endothelial cells (Bevilacqua et al, 1989). Recent developments in peptide chemistry and molecular biology yielded diverse peptide libraries consisting of numerous random peptide sequences (Pasqualini et al, 1996). Peptides with specific biological activity capable of mediating selective localization in tissues such as lung (Johnson et al, 1993) or lymphocytes (Cepek et al, 1994) have been obtained. An important example is the recently reported families of angiogenesis suppressing/inducing integrins that suppress or encourage the generation of new blood vessels (Varner et al, 1996; Folkman, 1996). These proteins are adhesion receptors not present in normal tissue but appear on endothelial cells of blood vessels of neovasculating areas. Since neovascularization is typical of malignant tissues at a certain stage, substances that interact with integrins might be considered as tissue markers for contrast agent delivery to blood vessels in neovasculating tumors (Brooks et al, 1994; Arap et al, 1998). Systematic screening of chemically-modified proteins (Neurath et al, 1995; Fujita et al, 1994) also yielded products recognized selectively by specific cells, for example, aromatic acid anhydrides that block CD4 cell receptors for HIV-1. Several systems were described that utilize macromolecular carriers armed with targeting ligands recognized by specific cell types (Monsigny et al, 1994; Hashida et al, 1995).

Tissue-targeting research and practice also utilize several alternative approaches. Some rely on physiochemical properties leading to passive uptake and accumulation, such as inherent accumulation of the agent by the target tissue (e.g., iodine by the thyroid). An important mechanism is the enhanced permeability and retention (EPR) phenomenon whereby molecules of a certain size may diffuse through blood vessels in areas of neovascularization as in malignant tissues (Matsumura et al, 1986; Duncan et al, 1996). Although this mechanism is not specific in terms of organ and type of the malignancy, a contrast agent may be delivered to all neovascularizing lesions by means of EPR, thus providing important pathological and anatomical information for many types of tumor.

~~The intense activity in the field of targeting~~
drugs to specific organs, tissues or cells have yielded a variety of carrier systems such as pro-drugs, liposomes, e.g., sterically stabilized liposomes (SSL) (Kedar et al, 1994) or polymers, both natural and synthetic. The carrier conveys the drug to the specific tissue (via antibody or a tissue marker) where the drug executes the therapy. Attempts to use selective delivery for diagnosis by targeting contrast agents are not as common, though MRI or sonographic agents are under examination. On the other hand, scintigraphy, based on the accumulation of radioisotopes in particular organs or in cancer lesions, is commonly practiced in clinics. X-ray absorbing agents, such as barium and iodine, are being used routinely in non-specific administration.

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SUMMARY OF THE INVENTION

It is an object of the present invention to provide

a delivery system and a method for tumor diagnosis, prognosis and follow-up of cancer therapy by using macromolecular carriers with or without specific tissue markers, for delivering heavy metals with an atomic number (hereinafter indicated by "Z") within the range 75-92, to selected organs or sites for tumor-enhanced X-ray imaging.

Another object of the present invention is to use the same delivery system and method for delivering lower Z elements which have the property of an abrupt discontinuity/ change in their X-ray attenuation coefficients, and whose attenuation threshold is within the X-ray energy range used for the specific radiography technology selected (e.g., mammography, computed tomography, digital radiography). Thus, the images obtained from two parts of the X-ray energy spectrum, one above the threshold and one below the threshold, can then be used to digitally generate a difference image having superior contrast. The two images can be generated either by filtering the impinging radiation or by analyzing the detected radiation according to the above threshold and the below threshold contributions. All elements with Z in the range of 33-50 progressively exhibit such a discontinuity/ change in X-ray attenuation coefficient in the 10-30 keV range within which all mammography is applied.

The present invention thus relates to a delivery system for targeting a contrast agent to specific organs for the purpose of tumor diagnosis, prognosticating the effectiveness of chemotherapy in treating cancer and for follow-up of cancer therapy by X-ray imaging, where the delivery system includes a conjugate or complex of a macromolecular carrier and a contrast agent selected from: (a) a compound of a heavy element with an atomic number in the range of 75-92, and (b) an element with a property of an abrupt change in its X-ray attenuation coefficient within the energy range used for radiography, where the macromolecular carrier may be optionally linked to a specific tissue marker.

The present invention further relates to methods for tumor diagnosis, for prognosticating the effectiveness of

chemotherapy in the treatment of cancer, and for follow-up of cancer therapy by X-ray imaging, which involves administering to a patient an effective amount of a delivery system of the present invention, followed by X-ray imaging of the patient.

- 5 As a non-limiting example, the delivery system is used for diagnosis, prognosis and/or follow-up of chemotherapy for breast cancer.

Abbreviations

- 10 The following abbreviations are used throughout the specification:

ADH: adipic acid hydrazide; BSA: bovine serum albumin; CDDP: cis-diamminedichloroplatinum II (cisplatin); CMdex: carboxymethyl dextran; DDW: double-distilled water; 15 EPR: enhanced permeability and retention; Hydr (H in the figures): hydrazine or hydrazide; OPDA: o-phenylenediamine; Ova: ovalbumin; St: streptavidin; TNP: 2,4,6-trinitrophenyl.

BRIEF DESCRIPTION OF THE DRAWINGS

- 20 Figs. 1A-1C show biodistribution (%/g tissue) of the radioiodinated degradable albumins BSA (Fig. 1A) and Ova (Fig. 1B) and the non-degradable St (Fig. 1C) with and without TNP modification (liver marker) in CD-1 male mice, as a function of time (15-120 min) following intravenous 25 injection. Levels of the native BSA, Ova and St and the modified TNP-BSA, TNP-Ova and TNP-St expressed as mean %/g tissue are depicted for blood (Bl) and six organs (heart, H; lung, Lu; spleen, Sp; liver, Liv; kidney, Kid; and bone).

- 30 Fig. 2 depicts the biodistribution (%/g tissue) of non-biodegradable iodinated TNP-St in tissues of CD-1 male mice as a function of time (14h-7 days). The levels in blood and six organs are shown, with the liver exhibiting long-term accumulation following injection into mice.

- 35 Fig. 3 shows the determination of OPDA concentration required for complete dissociation of CDDP from CMdex. CDDP, CDDP + dextran or CDDP + CMdex (6 µg CDDP per sample) were placed at 100°C for 3 minutes (to form the Pt-CMdex complex) and increasing amounts of OPDA were added.

Reaction with OPDA proceeded for 5 more minutes at 100°C and the absorbance at 703 nm was determined. A concentration of 3.8 mg/ml OPDA (or OPDA to CDDP ratio of 950:1) resulted in complete removal of CDDP from the dextran carrier.

5 Figs. 4A-4B show the evaluation of the interaction between CDDP and adipic acid dihydrazide (ADH) or hydrazine (H). Increasing amounts of ADH or hydrazine were reacted for 5 minutes at 100°C with a constant amount (6 µg) of CDDP when OPDA (4 mg/ml) was added for 5 minutes at 100°C. The
10 absorbance at 703 nm was determined (Fig. 4A). In Fig. 4B, the molar ratio of ADH or hydrazine bound per CDDP was calculated from the absorbance presented in Fig. 4A. More efficient interaction was observed with hydrazine.

Fig. 5 shows the determination of reversibly and
15 irreversibly bound Pt in CMdex and CMdex-Hydr complexes. CDDP (60 µg), CDDP + dextran, CDDP + CMdex or CDDP + CMdex-Hydr reacted for 3 min at 100°C at a molar ratio of 500:1. Each sample was then dialyzed for 12 hours in 1 ml volumes against 10 ml DDW. Samples from the dialyzed
20 material (in) and the dialysis DDW (out) were removed for interaction with OPDA (5 minutes at 100°C), and the absorbance at 703 nm was determined. Most of the CDDP complexed to CMdex was reversibly bound, whereas most of the CDDP bound to the CMdex-Hydr was irreversibly bound. CDDP
25 "lost" means CDDP not found by OPDA reaction inside or outside the dialysis bag, i.e. it could be lost or irreversibly bound.

Fig. 6 shows the biodistribution (%/g tissue) of CMdex-Pt before and after TNP modification at 24 and 56 hours
30 in various tissues. TNP modification resulted in specific long-term liver accumulation following injection of the TNP-modified CMdex-Hydr complexed to CDDP.

Figs. 7A-7B show mammography film images of a mouse treated with Pt-loaded CMdex-Hydr, 12 hours post
35 administration (Fig. 7A), and a control mouse treated by the same dextran derivative with no Pt load (Fig. 7B). The liver of the first mouse is darker and more clearly delineated,

whereas that of the second mouse is indistinguishable from other organs.

Fig. 8 shows a computed tomography (CT) image of a 1 mm-thick slice through the Plexiglas phantom. The various square compartments contain Pt, in aqueous solution, at concentrations (left to right) 0, 10, 30, 100, 300 and 1000 $\mu\text{g/ml}$. The corresponding average gray levels, measured within the circled area, are: 39.9, 36.1, 43.0, 51.3, 75.7 and 132.1. The effect of the Pt X-ray attenuation is clearly seen and can be quantified from 100 $\mu\text{g/ml}$ on. A thumbnail top view of this phantom is seen at the bottom left of the image. The dark oval shaped areas in the squares are air bubbles, excluded in the analysis. This data is presented in graphic form in Fig. 9.

Fig. 9 is a graphical presentation of the X-ray attenuation in the 0-1000 $\mu\text{g/ml}$ Pt Na_2PtCl_6 solutions shown in Fig. 8. Each group of bars corresponds to a given compartment of the phantom: a. actual CT values (dashed bars); b. normalized values (0 concentration value subtracted; dark bars); c. logarithmic presentation of b (open bars). At 100 $\mu\text{g/ml}$ sample, the increased X-ray attenuation is clearly detectable.

DETAILED DESCRIPTION OF THE INVENTION

The present invention was developed from a new concept based on simple physical principles relating to differential radiation attenuation by tissues of different densities. Such differences can be enhanced by chemical and biological means. The implementation of this concept of tumor-specific X-ray imaging, e.g., mammography, utilizes current radiographic equipment and will permit efficient tumor diagnosis, including population screening, with reduced radiation doses.

Physiological and pathological characteristics of an imaged tissue should offer vital information for tumor diagnosis. This is achieved according to the present invention by selectively loading the tumor with appropriate

contrast agents through physiological processes or tissue-specific markers. For X-ray radiography, the best contrast materials are high atomic number (heavy metal) elements which exhibit strong X-ray attenuation. The success of such an approach depends on the possibility of selectively conveying significant amounts of the contrast agent into the cancerous tissue. To reach this goal, the present invention provides a delivery system in which selected contrast agents are bound to carrier molecules that specifically accumulate in the cancer tissue.

For breast cancer diagnosis, specific tissue markers are preferred for targeting breast tissue, but since there are currently no such satisfactory markers, the invention presently uses as a preferred embodiment the general enhanced permeability and retention (EPR) phenomenon (Matsumura et al, 1986), known to induce carrier-ligand accumulation in neovasculating cancerous tissues (Duncan et al, 1996). The invention can also be used for tumor diagnosis in other tissues or organs, particularly when tissue-specific markers are unavailable. The present delivery system, however, can further incorporate specific tissue markers when they are available for imaging of selected tissues or organs.

The delivery of contrast agents to specific organs may benefit from the vast information regarding targeting and controlled release of drugs for therapy. However, there are significant differences between the two systems due to the different requirements, as shown in Table 1.

Table 1
Drug Versus Contrast Agent Targeting

	Drug Delivery	Contrast Medium Delivery
Release of Active Component	Required	Not Required
Intracellular Release	Often Required	Not Required
Controlled Release	Advantageous	Not Applicable
Long Maintenance	Advantageous	Not Advantageous
High Concentration within Tissue	Advantageous	Required
Repeated Administrations	Routinely (Often)	May Not Be Required
Attached Chemical	Chemotherapeutic Drug	Heavy Metal
Toxicity	Accepted up to a Certain Level	Unacceptable (for Screening)
Invasive Administration	Might Be Considered	Unacceptable

5 A transport system which is inadequate for drug delivery may prove suitable for contrast agent transport. Thus, for example, conditions such as long maintenance, controlled and intracellular release of the active component
10 are essential in drug targeting but are not required for diagnostics. On the other hand, a high concentration of the delivered agent for diagnostics is essential.

 The purpose of the present invention is both to improve the delineation of small-size cancerous tissue and to
15 provide a clear indication regarding its pathological status. These goals can be achieved by taking advantage of distinct functional and physiological properties of the cancerous tissue for targeting contrast agents, followed by X-ray imaging.

20 The delivery system according to the present invention, developed for delivering a contrast agent to a target tissue or organ for X-ray imaging, includes a conjugate or complex of a macromolecular carrier and a

contrast agent, where a specific tissue marker can be optionally linked to the macromolecular carrier when available for a specific tissue or organ.

The contrast agent may be any non-toxic (in free or in bound form) organic or inorganic compound of a heavy element with atomic number $Z = 75-92$, e.g., heavy metals such as Pt, Au, Tl, or alternatively, an element with an X-ray attenuation threshold in the relevant energy range for the specific radiography technology, e.g., Zr, in the case of mammography. At the X-ray attenuation threshold, there is an abrupt change or discontinuity in the X-ray attenuation coefficient with respect to X-ray energy. All elements with Z in the range of 33-50 exhibit such an X-ray attenuation threshold in the 10-30 keV X-ray energy range. When an energy range above 30 keV is used in radiography, an element with Z above 50 is used for its property of having an X-ray attenuation threshold in the appropriate X-ray energy range.

The heavy element is preferably a heavy metal selected from Pt, Au and Tl, and the macromolecular carrier is selected from liposomes and natural or synthetic polymers.

The heavy metal compound may be attached to the natural or synthetic polymer through a metal-binding group or ligand containing nitrogen, nitrogen-oxygen or sulfur atoms, such as thiol, hydrazido, piperazine and the like, or the heavy metal compound may be complexed to the polymer through a metal-chelating ligand, such as iminodiacetic acid, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminopentaacetic acid (DTPA), hydroxyquinoline, hydroxamic acids, hydrazides and the like, and combinations thereof.

Potential detection techniques include, but are not limited to, 2D digital radiography, CT, dual energy and image subtraction, "positive" and "negative" imaging, etc, all based on X-ray attenuation in the imaged body. An X-ray detection means include X-ray filter and screen-enhanced film and digital X-ray detectors.

Selection of the appropriate contrast agent and its delivery system is an apparent prerequisite according to the invention. Since there is a profound increase in X-ray

attenuation with an increase of the atomic number (Z), it is advantageous to select heavy elements of $Z = 75-92$ as preferred contrast agents rather than the more common iodine (I). Metals of choice are gold and platinum which are already in use in several pharmaceutical formulations. In case of dual energy, however, other elements having lower Z might be preferred, due to the abrupt change in their X-ray attenuation coefficient within the relevant energy interval.

The metal-loaded macromolecular carrier can be specifically targeted to certain types of cells, e.g., tumor cells, delineating primary tumor growth or tumor metastases by "positive imaging", or to the tumor-bearing organ, delineating tumor lesions by "negative imaging". Antibody-directed contrast agent targeting may be used wherever specific and effective antibodies are available.

Two types of macromolecular carriers can be used for heavy metal contrast agents according to the present invention. The first is a conjugate of the polymer with a metal binding ligand to which a heavy metal is attached. This general type is preferable for non-targeted delivery into cancerous sites by physical entrapment processes, such as those occurring in neovascularization sites of tumors. The second type is a conjugate of the polymer with a targeting ligand (tissue marker), in addition to the metal binding ligand to which a heavy metal is attached. The role of the targeting ligand is to direct the whole conjugate to a specific tissue via the affinity interaction of the targeting ligand to a given epitope on the specific cell. Certain targeting ligands/tissue markers are known, as described in Table 2.

Table 2
Tissue Markers (Examples)

Tissue	Marker	Reference
Liver Hepatocytes (Asialoglycoprotein Receptor)	Gal, GalNac	Ashwell et al (1982)
Liver Kupffer Cells	Man, GluNac, Fuc	Taylor et al (1992)
B Cells (IgE Fc Receptor)	Gal	Kikutani et al (1986)
Leukocytes (L-selectin)	Fuc, Sialic acid	Lasky et al (1989)
Endothelial Cells (E-selectin)	Fuc, Sialic acid	Bevilacqua et al (1989)
Platelets (P-selectin)	Fuc, Sialic acid	Johnston et al (1989)
Lung and Liver Macrophages	Man, Fuc	Taylor et al (1992)
Lung	Endothelial Marker	Pokutta et al (1994)
Brain ¹	Ser-Arg-Leu (SRL) Containing Peptide	Pasqualini et al (1996)
Kidney Blood Vessels	CLPVASC Peptide	Pasqualini et al (1996)
Tumor Blood Vessels	Arg-Gly-Asp (RGD) and Asn-Gly-Asp (NGD) Containing Peptides	Arap et al (1998)
CD4 Cell Receptors for HIV-1	3-hydroxyphthalic and trimellitic anhydrides	Neurath et al (1995)

5 ¹ Many cell adhesion receptors recognize simple sequences
that can be reproduced as synthetic peptides homing to
these cells. Peptides capable of binding to cell adhesion
receptors, such as integrins, can be used as mediators for
10 cell or tissue targeting. Factors such as EGF, VEGF, MHS,
etc. can also serve for specific tumor targeting to the
specific receptors (for these factors) that are
overexpressed on certain tumor cells.

15

A tissue marker in addition to those of Table 2, is
the 2,4,6-trinitrophenyl (TNP) group, a targeting ligand to
the liver. As described in Schechter et al, 1996, and in PCT

Publication No. WO 97/22879 of the same applicants, TNP-streptavidin, obtained by reaction of ϵ -amino groups of lysine residues of streptavidin with 2,4,6-trinitrobenzenesulfonic acid (TNBS), accumulates mainly in the liver.

5 In order to select a suitable delivery system according to either type of targeting system described above, biodistribution of the metal-macromolecular carrier conjugate provides information regarding its specific sites of accumulation, metal uptake kinetics and saturation. To
10 provide biodistribution data, the polymer, e.g. dextran or a dextran derivative such as CMdex or CMdex-hydrazide, is charged with several tyrosyl residues for radioiodination, the radiolabeled agent is then administered intravenously into mice, and at set time intervals, blood and organs are
15 analyzed for biodistribution parameters (Schechter et al, 1996). Determination of the actual metal accumulation can be done by atomic absorption spectroscopy, XRF (X-ray fluorescence) and ICPS (inductively-coupled plasma spectroscopy) analysis of tissue samples. Experiments are
20 directed at both tissue accumulation for "negative imaging" and tumor accumulation for "positive imaging".

The preferred systems for delivery of heavy metal contrast agents according to the present invention are macromolecules carrying a heavy metal as defined in the
25 present invention. Such a macromolecule that will be defined for the purpose of this invention as a "macromolecular system" is composed of (a) a suitable macromolecular carrier, (b) a metal binding group as a pendant group, and (c) a heavy metal salt or complex. The metal salt or complex will be
30 part of the macromolecular system due to attachment via the metal binding ligand. The scientific literature, including patents, teaches many ways to bind a metal salt or metal complex to a macromolecular carrier, including the present inventors' own publications (Warshawsky, 1980, 1982, 1984,
35 1986, 1987a, 1987b, 1987c) which show how to bind platinum and platinum group metal (including gold) to polymeric carriers. The literature also teaches us how to bind metal ions to polymers for pharmaceutical uses (Dunn et al, 1991).

Of particular interest for the present invention are polymer carriers, such as, for example, carbohydrates, e.g. dextrans and modified dextrans such as carboxymethyldextrans, polyamino acids such as poly-L-lysine and polyglutamic acid, the polymers described in Duncan et al (1996) and Pimm et al (1996), polymers which display selective delivery or accumulation in the target tissue, e.g. polymethacrylic acid in neovasculating regions such as in tumors, as well as polymers modified with specific tissue markers, including address molecules with known tissue or organ specificity.

The present invention relies on the use of macromolecular carriers that are already described in the literature and are used in practice, provided that they conform to the criteria for contrast medium delivery described in Table 1 for diagnostic applications, particularly the criteria of high metal loading and very low systemic toxicity. The present inventors have found that, although sometimes the right compositions are known, they lack sufficient numbers of functional groups to allow binding of a sufficient amount of the heavy metal. The present invention shows how to achieve this high loading in the particular cases given. Other synthetic molecules of preselected shapes, such as liposomes or ball shaped structures such as "dendritic" structures, are also included. Such dendritic structures comprise a polymeric carrier such as carboxymethyldextran onto which radially branching appropriate bifunctional molecules, e.g. dicarboxylic acids and/or hydrazides thereof, are anchored, thus providing a way to increase the "per unit" loading of functional metal binding groups and allow for higher contrast agent load so that the total carrier dose can be decreased. It also provides for a way to control or manipulate the size and shape of the macromolecular system and to engineer optimal site-specific delivery by controlling the sieving effect as in neovascularization sites in active tumors.

Since no toxic side-effects are tolerable, particularly in large population screening, such as in mammography, stringent measures should be taken to ensure

complete safety. Thus, the metal carrier agents according to the invention are characterized by high-affinity bound metals by chelates or non-dissociable binding, e.g., covalent binding, to prevent metal release. Preferred macromolecular carriers are those that form non-toxic conjugates or complexes with the metal. Ultimately removable or biodegradable polymers should be used to ensure limited in vivo residence time and complete clearance. The macromolecular carrier should not be toxic either by itself or in its conjugated or complexed form, and its properties should be a compromise between circulation of sufficient duration to allow accumulation at the tumor site prior to X-ray imaging and subsequent efficient removal to ensure limited in vivo residence time and complete clearance. For this purpose, suitable candidates are sterically stabilized liposomes and various macromolecules, such as dextrans and synthetic polymers, e.g., polymethacrylic acid, a polymer which has been studied thoroughly in targeted drug delivery and radioactive diagnostic research (Duncan et al, 1996; Pimm et al, 1996). The chemical binding between the contrast agent and the macromolecular carrier should be strong enough to prevent metal release to avoid the poisonous effects of the heavy metal and to allow high agent loading capacity to enable minimal carrier doses.

According to the present invention the amount of agent, e.g., gold or Pt, to be delivered to the tissue, required for sufficient contrast enhancement with current X-ray radiographic or tomographic equipment, is of the order of 100 $\mu\text{g/ml}$ (or per gram tissue) or more. Depending on the efficiency of the targeting, this imposes a lower limit on the amount of agent the conjugate should contain. Considering the level of concentration reached in our preliminary experiments on a model system of liver targeting, i.e., 60-70 % injected dose/ml tissue, the amount of agent in the conjugate should exceed 160 μg per injected dose. The actual amounts are to be adjusted according to the delivery system to be selected.

Based on our previous studies on carrier-mediated

drug targeting (Schechter et al, 1986a, 1986b, 1987a, 1987b, 1989a, 1989b, 1991, 1995; Arnon et al, 1989), dextran was selected as a preferred multi-purpose carrier for tissue-specific targeting. Dextran is available in different molecular sizes, and it is characterized by high solubility in water, resistance to proteolytic degradation, and low immunogenicity. Dextran is also non-toxic, as known from extensive experience in clinical use of dextran as a plasma expander (Larsen, 1989), and can be chemically modified. For instance, dextran modified to the carboxymethyldextran form (CMdex; Hurwitz et al, 1980) is highly substituted with carboxyl groups that can be further derivatized with other groups. Dextran has been shown to be modified for attachment to antibodies in immunotargeting system (Schechter et al, 1987b; Arnon, 1989; Aboud-Pirak et al, 1989), to carry different drugs (Schechter et al, 1986a, 1986b, 1989a, 1995; Hurwitz et al, 1980; Bernstein et al, 1987), biotinyl groups in avidin-mediated procedures (Schechter et al, 1996, 1991) and tyrosyls for radioiodination (Schechter et al, 1996). Due to the abundance of functional groups, modified dextrans can serve as backbone carriers for chemical attachment of tissue markers and still carry enough groups for coupling or complexing the delivered agent.

One aspect of the present invention is aimed at preparing a targeting system for tissue-selective delivery of metals that can serve as contrast agents for X-ray radiography. Standard available radiographic techniques can be improved by conveying significant amounts of the contrast agent, preferably elements with high atomic numbers, such as Pt, into the cancerous tissue. This is expected to increase the density differences between malignant and normal tissue. Previous experience with Pt compounds or complexes between CDDP, a known chemotherapeutic drug, and macromolecular substances, e.g. CMdex (Schechter et al, 1986a, 1986b, 1987a, 1987b) enabled the present inventors to design a Pt-carrier system for contrast agent delivery. Such a delivery system is based on (a) a carrier, e.g. CMdex-hydrazide, enriched with functional groups to allow for heavy metal, e.g. Pt,

loading capacity as well as tissue marker, e.g. TNP, conjugation; and (b) strong chemical binding between the metal and the carrier to prevent toxic metal release.

5 According to the present invention, the contrast agent delivery system is chosen so as to improve cancer detection capability of existing X-ray imaging techniques and procedures, and in order to provide higher tumor-specificity and better resolution. This should permit efficient detection of small cancer lesions at lower radiation doses.

10 The improvement of cancer detection capability by tumor-specific X-ray imaging according to the invention will lead to earlier and more conclusive diagnostic procedures for solid tumors that will have significant impact on the prognosis of patients and will permit more efficient
15 ~~non-invasive follow-up of cancer patients subjected to~~ therapy as well as a better control of drug delivery to tumors.

20 Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLES

25 In the Examples, the following materials and methods are used:

MATERIAL AND METHODS

30 i) **Materials.** Streptavidin (St) was provided as a gift from Boehringer-Mannheim GmbH (Mannheim, Germany), and CDDP from Abic Company (Ramat Gan, Israel). CMdex was prepared from dextran as previously described (Hurwitz et al, 1980). N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethyl-amino-propyl)-carbodiimide (EDC), 2,4,6-trinitrobenzenesulfonic acid (TNBS), dicyclohexylcarbodiimide (DCC), t-butyloxycarbonyl-
35 tyrosine (BOC-Tyr), and 2-[N-morpholino]ethanesulfonic acid (MES) buffer were purchased from Sigma (St. Louis, MO). O-phenylenediamine (OPDA) was obtained from Fluka Ag (Buchs, Switzerland), and N,N-dimethylformamide (DMF) from Merck-

Schuchardt (Munich, Germany).

CMdex Products: Dextran Mr-10, 20, 40, 70 or 250 was converted into the carboxymethyl form CMdex according to a procedure previously described for dextran Mr-40 (Hurwitz et al, 1980). Dextran was reacted with chloroacetic acid in a strongly basic solution and the products were analyzed by NaOH titration for the degree of carboxyl group content. Samples of the Na-salt derivatives obtained after synthesis (10 mg) were dissolved in 1 ml 0.3M HCl to convert the COONa groups into COOH, dialyzed against 2L double distilled water (DDW) and lyophilized. Dry samples of the Na-free products (5 mg), dissolved in 2 ml DDW, were titrated with 0.01M NaOH in the presence of phenolphthalein, and the carboxyl group content (carboxymethyl substitution) and the molecular weight of the CMdex derivatives were calculated (Table 3). Dextran Mr-10 gave rise to a 12.2 kDa CMdex product containing 0.63 carboxyl groups per glucose unit of 40 carboxyls per dextran. Ratios of carboxymethyl/glucose in the different preparations ranged between 0.38 to 1.2. CMdex Mr-20, 40, 70 and 250 contained 47, 275, 297 and 1875 carboxyl groups, respectively.

Table 3
CMdex Mr-10, 20, 40, 70, 250

Dextran kDa	COOH/Unit	Unit Da	CMdex KDa
10	0.63	198	12.2
20	0.38	184	22.6
40	1.10	226	55.8
70	0.68	204	88
250	0.73	231	356

ii) Preparation of CMdex-hydrazide (CMdex-Hydr)

CMdex-hydrazide was prepared from dextran Mr-250 according to two different protocols. Protocol 1 produced a CMdex-hydrazide product containing many hydrazide and few free carboxyl groups and did not bind Pt well, while the

product of Protocol 2 contained few hydrazido and many free carboxyl groups and bound Pt effectively.

Protocol 1: CMdex (30 mg) was dissolved in 0.15M MES buffer pH 5 (1.5 ml), and aqueous hydrazine (0.5 ml, 5M), adjusted to pH 6 with 6M HCl, was added. Then, EDC (53 mg x 3 at 20 min intervals) was added while vortexing. After 7 hours at room temperature (RT), the solution was dialyzed against 2 x 4L DDW for 12 hours. The sample was then treated with 6M HCl (0.1 ml) and dialyzed as described above. The product CMdex-Hydr was obtained as 3.5 ml of a clear solution of pH ~7.

Protocol 2: CMdex (30 mg) was dissolved in 1.5 ml 0.15M MES buffer pH 5. NHS (100 mg) was added, followed by the immediate addition of 160 mg EDC. After mixing for 1 minute, the reaction mixture was added to hydrazine (0.5 ml of 6.6M at pH 8) while vortexing. The solution was then treated as described above. The product CMdex-Hydr was obtained as 5 ml of a slightly turbid solution (5 ml) of pH ~4 (which became clear at pH 7).

20 **iii) Synthesis of "dendritic molecules" from CMdex:**

Dendritic molecules were synthesized by reacting CMdex (50 mg) with iminodiacetic acid (580 mg) in the presence of EDC (200 mg) to increase the carboxyl group content. Hydrazine was coupled to the dialyzed product by adding NHS (115 mg), EDC (400 mg) and hydrazine (1.8 ml of 6.6M) as described in section (ii) above, Protocol 2, for CMdex-Hydr. The dendritic product contained 600 hydrazide groups/dextran.

30 **iv) Quantitative determination of dextran**
(according to Dubois et al, 1956): CMdex-Hydr (50-150 µg dextran range) in 0.8 ml DDW was mixed with 5% phenol solution (0.5 ml in a 100 x 12 mm glass test tube) and 2 ml concentrated sulfuric acid was added. The reaction mixture was vortexed immediately, and the absorbance at 485 nm was determined 10 minutes later. The concentration was calculated relative to standard CMdex-Hydr solutions.

35 **v) Quantitative determination of hydrazide groups.**

CMdex-Hydr (50-150 µg dextran range) in 1 ml 0.05M NaHCO₃ was reacted with 250 µg TNBS (25 µl of 10 mg/ml). The

absorbance at 500 nm was determined after 10 minutes, and the concentration of hydrazide groups was calculated relative to standard solutions of adipic dihydrazide.

vi) Quantitative determination of carboxyl groups.

- 5 CMdex-Hydr (3-5 mg dextran range) in 2 ml DDW was titrated with 0.01N NaOH in the presence of phenolphthalein.

- 10 **vii) Preparation of tyrosylated CMdex.** CMdex (55 mg) was dissolved in 2 ml DDW, NHS (7 mg) was added, followed by the immediate addition of EDC (30 mg). After mixing for 1 minute, tyrosine (2 mg) was added while vortexing. The reaction continued for 15 minutes at RT, and the solution was dialyzed against DDW. The molar substitution ratio of tyrosine, as determined from the absorbance at 280 nm in reference to non-tyrosylated CMdex, was 20:1.

- 15 ~~**viii) Preparation of CDDP complexes with**~~
CMdex-Hydr. CDDP in DDW (3 mg/ml, dissolved by slight warming) was mixed with the CMdex-hydrazide derivative at a molar ratio of 500:1. The reaction mixture was placed in a 100°C water bath for 3-5 min and was further treated as
20 described below in section ix.

- ix) Quantitative determination of CDDP** (according to Golla et al, 1973): Samples of undialyzed and/or dialyzed CDDP-CMdex-hydrazide complexes of section viii above containing estimated amounts of 3-10 µg of CDDP/0.6 ml DDW
25 were mixed with 0.6 ml OPDA dissolved in DMF (4 mg OPDA/ml DMF, or as indicated). The reaction mixtures were placed in a 100°C-water bath for 3-5 minutes (as indicated), and the absorbance of the light blue colored solutions formed due to interaction of CDDP (free or reversibly-bound to CMdex) with
30 the OPDA ligand was determined at 730 nm after cooling. The amount of CDDP in the reaction mixture that reacted with OPDA was determined in reference to standard solutions of free CDDP. All CDDP values refer to the OPDA-reactive material, i.e., reversibly-complexed CDDP that is capable of being
35 released from the carrier in favor of the OPDA ligand.

x) TNP modification. The reaction was carried out as described in WO 97/22879, as follows: TNBS dissolved in

DDW (10mg/ml) was reacted at a molar ratio of 200:1 with St, Ova and BSA (1 mg/0.5 ml, 0.2M sodium bicarbonate). After 20 min at room temperature, the pH was adjusted to 7.3 and the products TNP-St, TNP-Ova and TNP-BSA were dialyzed.

5 CMdex-hydrazide was similarly modified with TNBS to produce TNP-CMdex-hydrazide. CMdex (Mr 250 kDa, 1875 carboxyl groups/dex) was substituted with hydrazido groups according to Protocol 2, and the product was reacted with TNBS at 120:1 molar ratio to form TNP-substituted
10 CMdex-hydrazide. The TNP-CMdex-hydrazide was then reacted with CDDP (2-3 min at 100°C, followed by dialysis to remove uncomplexed Pt) to produce a polymer carrying 60 mol TNP and up to 300 mol complexed Pt/mol CMdex-hydrazide.

~~15 (xi) Radioiodination. The ¹²⁵I-proteins or ¹²⁵I-Tyr-TNP-CMdex-Hydr were produced by reaction with ¹²⁵INa as described in WO 97/22879.~~

EXAMPLE 1: Biodistribution studies with unmodified and TNP-modified BSA, Ova and St

20 This experiment was conducted with the enzyme-degradable albumins BSA and Ova and the enzyme-resistant protein streptavidin. Groups of 10-12-week old CD-1 male mice were given intravenous injections into the lateral tail vein of the radioiodinated derivatives BSA, Ova,
25 St, TNP-BSA, TNP-Ova and TNP-St (1-2 µCi/mouse). At set time intervals, blood samples were withdrawn from the tail, the mice were killed and their organs were dissected out, blotted dry, weighed and counted for radioactivity. The results are expressed as mean percent of injected radioactivity dose/gram
30 tissue (%/g) or per whole organ (liver).

Figs. 1A-1B show that, when tested at a time range of 15-120 min, TNP modification of BSA and Ova led to rapid hepatic uptake as compared to unmodified BSA and Ova. However, as described before in WO 97/22879, at the 2-hour
35 time point, both BSA and TNP-BSA were down to <0.3%/g in blood and organs and both Ova and TNP-Ova were down to <0.6%/g, while the biodistribution profile of TNP-St tested at a time range of 15-180 min, was different (Fig. 1C): blood

levels for both TNP-St and St were kept high for a longer period of time (22-33%/g) whereas levels in other tissues, including the liver, were low (<10%/g). Elevation of the St in the kidney and TNP-St in the liver was delayed and started to build up at 120 min (23% and 18%/g, respectively), indicating that the TNP modification of proteins increases their uptake by the liver. The slow degradation and processing of Sand TNP-St leads to retardation of uptake and to subsequent accumulation in the target organs (St in kidney and TNP-St in liver).

Fig. 2 shows that monitoring the level of TNP-St in different tissues in a period from 14h to 7 days, 33%/g retention of TNP-St was found in the liver at 14h which increased to 42%/g at 24h and slowly declined later on.

As already shown by the present inventors (Schechter et al, 1996), TNP modification of proteins or polymers results in high liver accumulation: degradable molecules, e.g., albumins, exhibited short-term accumulation (Figs. 1A-1B), whereas non-degradable molecules, e.g., enzyme-resistant proteins such as streptavidin (Figs. 1C, 2), led to long term liver retention.

EXAMPLE 2: Selection of CDDP-binding groups

CDDP complexes of CMdex-hydrazide were characterized in comparison to CDDP complex of CMdex (all derived from dextran Mr-250). The OPDA reagent was used for estimation of the amount of bound CDDP and the relative binding affinities (releasable vs. non-releasable Pt) between CDDP and the different dextran carriers under defined conditions. Since CDDP binds to CMdex in a reversible (releasable) manner, OPDA was first titrated to determine the OPDA/CDDP ratio required for complete removal of CDDP from the CMdex carrier. CDDP was reacted with CMdex at a molar ratio of 500:1 (3 min, 100°C) to form the CDDP-CMdex complex. Increasing amounts of OPDA were then added (5 min, 100°C) and the absorbance at 703 nm was determined in comparison to CDDP reacted with unmodified dextran. As shown in Fig. 3, at low

OPDA concentration (low OPDA/CDDP ratio), most of the CDDP (6 µg per sample) was bound to CMdex (low A703). Increasing OPDA concentration led to increased release of CDDP from the CMdex backbone in favor of OPDA (higher A703). At 3.8 mg/ml OPDA, or at a molar excess of 950 OPDA/CDDP, all of the Pt was "pilled off" the CMdex carrier in favor of OPDA. No interaction occurred between CDDP and unmodified dextran (high A703 nm, similar to that of free CDDP). This type of Pt determination provides the conditions required for complete removal of reversibly-bound CDDP.

Several amine-containing compounds were considered as possible high-affinity CDDP ligands for attachment to dextran. O-phenylenediamine (OPDA), which forms a colored complex with CDDP (absorbance at 703 nm), was found useful in determining relative binding affinities between CDDP and various ligands (Schechter et al, 1989b). Evaluation of two compounds, adipic dihydrazide (ADH) and hydrazine (Hydr), is shown in Figs. 4A and 4B. ADH or hydrazine were reacted at increasing molar ratios (5 minutes, 100°C) with CDDP (6 µg), and OPDA 4 mg/ml) was then added (5 minutes, 100°C). Complete complexing of CDDP to ADH and hydrazine was observed at a molar excess of 50:1 (no color reaction with OPDA). As molar excess of ADH and hydrazine to CDDP decreased, more Pt interacted with OPDA (increased color) up to a leveling-off ratio of 20 ADH:CDDP and 10 hydrazine:CDDP. This is the reason why hydrazine was selected for coupling to CMdex, namely due to its more effective interaction, i.e., lower release of Pt in favor of OPDA and lower ratio of hydrazine:CDDP interaction.

CMdex-hydrazide derived from dextran 250 (1875 carboxyl groups/dextran) was prepared according to Materials and Methods, section (ii), Protocol 1, giving rise to a highly substituted product, with 700 hydrazide groups per dextran or 37% of total carboxyl group content (Table 4). The pH of the product solution after HCl treatment and dialysis was around 7, indicating low carboxyl content. This was verified by NaOH titration which showed that only 20% of

the total carboxyls (370 mol/mol CMdex) were free, i.e., 800 carboxyl groups that were not substituted by hydrazide groups, were blocked.

5 CMdex-hydrazide derived from the same dextran 250 but prepared according to Materials and Methods, section (ii), Protocol 2, was less effective in terms of hydrazine substitution and contained 240 hydrazide groups per dextran (13% of carboxyls), but blocking of unsubstituted carboxyl groups did not occur, i.e., 1600 carboxyls (86%) were free.
10 The pH of this product following HCl treatment and dialysis was around 4.

Blocking of carboxyl groups during synthesis according to Protocol 1 with EDC alone could be attributed to cross-linking by hydrazine, but different synthetic
15 ~~procedures showed that increasing hydrazine molar excess to~~ prevent cross-linking was ineffective. Blocking could, therefore, occur by EDC itself, which does not take place in the presence of NHS (Protocol 2). The protecting effect of NHS on EDC blocking of carboxyls could be demonstrated when
20 CMdex alone (no hydrazine) was subjected to treatment with EDC in the presence or absence of NHS; only 40-46% of the carboxyl groups were free when NHS was left out, whereas 80-90% free carboxyls were free when NHS was included (Table 4).

Table 4
Synthesis of CMdex-hydrazide

	CMdex (mg)	EDC (mg)	NHS (mg)	Hydrazine (mmol)	H/dex (%)	COOH/dex (%)
1 ^a	30	160	-	2.5	37.8 [700:1]	20
2 ^b	30	160	100	3.3	13 [240:1]	86
3	10	50	-	-	-	40
4	10	50	60	-	-	80
5	10	100	-	-	-	46
6	10	100	30	-	-	90

a Protocol 1; b Protocol 2

The two synthetic procedures described in Table 4 gave rise to two different CMdex-Hydr products. An experiment, which results are presented in Table 5, was designed to characterize these two CMdex-Hydr preparations with respect to their irreversible (strong) CDDP binding. CDDP was reacted with dextran, CMdex and CMdex-Hydr, as described above, to form the corresponding CDDP-dex complexes, and part of each reaction mixture was dialyzed against DDW (4 hours, 2 x 2L). OPDA reactivity was determined on undialyzed and on dialyzed material. As shown in Table 5, when CDDP was reacted with dextran (Mr-250) at a molar ratio of 500:1 (3 min at 100°C), all of the CDDP (99%) was OPDA-reactive (4 mg/ml OPDA) before dialysis and none after dialysis.

Table 5
Characterization of CMdex-H-Pt Complexes
Dex 250. CDDP:dex=500:1 (100 µg Pt)

Dextran Derivative	CDDP before Dialysis (%)		CDDP after Dialysis (%)		CDDP:CMdex Bound (molar ratio)	
	OPDA Reactive ^a	Irreversibly Bound ^b	OPDA Reactive ^c	Non-bound ^d	Reversibly Bound	Irreversibly Bound
Dextran	99	1	1	98	5:1	5:1
CMdex	95	5	87	8	435:1	25:1
CMdex-H Protocol 1 (700H/dex)	90	10	3	87	15:1	50:1
CMdex-H Protocol 2 (280H/dex)	54	46	4	50	20:1	230:1
CMdex-H Protocol 2 (100H/dex)	63	37	24	39	120:1	185:1
CDDP 100	100	0	1	99		

a. Complex was formed by reacting CDDP and dextran derivative at 100°C for 3 min and testing samples with OPDA at 100°C for 5 min. Represent percent reversibly + non-reversibly bound CDDP.

b. Values represent the difference between 100% CDDP input and % OPDA-reactive CDDP.

c. Reversibly bound CDDP.

d. Values represent the difference between OPDA reactive CDDP before and after dialysis.

This indicates that no binding occurred between CDDP and unmodified dextran and that dialysis was complete (no non-specific binding). When reacted with CMdex, a major fraction of the CDDP was reactive with OPDA before dialysis (95%). This means that either none of the CDDP was complexed and/or the CDDP was bound in a reversible manner. After dialysis, 87% reacted with OPDA, i.e., 87% (435 Pt/dex) of the CDDP was complexed to CMdex in a reversible manner (5% was

irreversibly bound, and 8% was free and removed during dialysis). When CDDP was reacted with CMdex-Hydr (Protocol 1; 700 Hydr/dex), 90% was OPDA-reactive before dialysis, i.e., 10% was irreversibly bound (50 Hydr/dex). After dialysis, only 3% was OPDA-reactive. This means that CMdex-Hydr prepared according to Protocol 1 did not bind CDDP effectively neither in the reversible nor in the non-reversible form. When CDDP was reacted with CMdex-Hydr, Protocol 2 (280 Hydr:dex), 54% was OPDA-reactive before dialysis, i.e., 46% was irreversibly bound (230 Hydr:dex). After dialysis, only 4% was OPDA-reactive, i.e., none of the CDDP was bound in a reversible manner. A CMdex-Hydr derivative of lower Hydr content (100 Hydr/dex) contained a lower fraction of stably-bound CDDP (37%) and a higher fraction of reversibly-bound CDDP (24%). It is concluded that: (a) contrary to the expected, binding of CDDP to the hydrazide-rich product was low, whereas binding to the hydrazide-low product, relative to hydrazine content, was good; (b) above a certain hydrazine substitution ratio, and despite the expected abundance of free carboxyl groups, there is no reversible binding of CDDP; and (c) reversibly-bound CDDP can be determined only in the dialyzed complex, whereas irreversibly-bound CDDP can be determined only on the undialyzed complex. It should also be noted that there was no simple direct correlation between the number of hydrazide groups and CDDP binding, the later being often higher.

In order to further determine the reversibly and irreversibly-bound Pt in CDDP-CMdex and CDDP-CMdex-hydrazide complexes, CDDP (60 µg), CDDP + dextran, CDDP + CMdex or CDDP+CMdex-Hydr complexes were each dialyzed against DDW (10 ml/sample) for 12 hours, and OPDA-reactive CDDP was determined in the dialyzed samples, as well as in the dialysis water. In this type of experiment, where relatively low amounts of CDDP are used and a long dialysis period is required, a fraction of the total free CDDP is lost (around 40%) due to non-specific complexing of CDDP to surrounding components such as dialysis bag, glass, etc.

As shown in Fig. 5, Pt determined for free CDDP or

CDDP reacted with dextran was 45-50% lower than the original Pt input. Only 12% loss of Pt was observed with the CDDP-CMdex complex. This can be expected since CDDP was CMdex-complexed and most of it (85%) was retained as reversibly bound Pt within the dialyzed material. This is in accord with our previous observation that CDDP complexed to CMdex is protected against irreversible interaction with body constituents (proteins, etc.) when injected into mice (Schechter et al, 1989a). When CDDP was complexed to CMdex-H (prepared according to Protocol 2), only a total of 14% of the CDDP input was found by measuring samples from inside and outside the dialysis bag. Assuming that Pt loss in this case is no higher than with the CMdex complex, it can be calculated that out of the 86% missing Pt, about 75% was irreversibly bound.

EXAMPLE 3: Metal targeting to tissues: Biodistribution studies with unmodified CDDP-CMdex-hydrazide (Pt-CMdex) and TNP-modified CDDP-CMdex-hydrazide (Pt-TNP-CMdex)

The system used for demonstrating Pt targeting to the liver consisted of complexes between CDDP and hydrazido-substituted polymers. CDDP can form high affinity, non-dissociable and pharmacologically inert complexes with certain polymers (Howe-Grant et al, 1980) such as dextran-hydrazide. Modifying dextran-hydrazide with the liver marker trinitrophenyl groups (TNP), as described in PCT Patent Appln. No. WO 97/22879 of the present applicants, leads to selective delivery to the liver with levels of up to 70 percent of the injected dose per gram tissue (%/g) or up to 80% of the total dose accumulated in the whole liver. This is based on studies (Schechter et al, 1996) showing that TNP modification of proteins or polymers resulted in high liver accumulation: degradable molecules, e.g., albumin, exhibited short-term accumulation (Figs. 1A, 1B), whereas non-degradable molecules, e.g., enzyme-resistant proteins such as streptavidin or dextran-hydrazide, led to long term liver

retention (Figs. 1C, 2, 6). Since accumulation is correlated with marker density, macromolecules can serve as targeting vehicles for diagnostic agents, provided their content of functional groups is high enough to carry both a sufficient quantity of marker molecules and the delivered agent.

Experiments were conducted on Pt delivery to mouse liver to demonstrate (a) the ability for loading a substantial amount of the contrast agent onto the carrier; (b) high specific accumulation of the complex in the target tissue; and (c) detectability of the agent loading by standard X-ray mammographic equipment.

For targeting the CDDP-CMdex-hydrazide complex to the liver via TNP, trace-tyrosylated CMdex (20 tyrosyl/dex) prepared according to Materials and Methods, section vii, was used to enable radioiodination with iodine-125 (Materials and Methods, section xi). Tyrosyl-CMdex was substituted with hydrazine and the Tyr-CMdex-hydrazide was treated with 120 mol TNBS/mol CMdex to obtain a TNP-modified CMdex-hydrazide containing 60 mol covalently bound TNP/mol dex (after dialysis). CDDP was complexed to the product (500:1 molar ratio, 3 min at 100°C followed by dialysis). A control complex was similarly prepared but without the TNP.

Mice were injected intravenously with 50 µg of the radioiodinated materials (Pt-TNP-CMdex and Pt-CMdex) and the mice were killed at 24 and 56 hours. Radioactivity in blood and organs was monitored in reference to tissue weight and biodistribution in terms of percent of total injected dose/gram tissue (%/g) was determined. As shown in Fig. 6, levels of I^{125} -CMdex-Pt (no TNP) in most tissues were in the range of 3-12%/g (22%/g in blood) at 24 hours after injection and were lower in most organs after 56 hours. The biodistribution pattern of the TNP-carrying complex was different: levels in blood and most organs was below 2%/g, except for the liver, where values reached 66%/g (82%/total liver) after 24 hours and were still at the same range at 56 hours. Spleen levels were also increased (23%/g).

The biodistribution and liver accumulation of radioiodinated TNP-dextran hydrazide loaded with Pt, at 24 and

56 hours following intravenous injection into CD-1 mice, is shown in Fig. 6.

In another experiment, the same complex (150 µg) was similarly injected (non-radioiodinated), and 12 hours later, the mice were sacrificed and their livers were removed.

5 Tissue extracts were prepared by homogenizing organs in 5 x vol of 0.1N NaOH/tissue weight for 60 seconds, followed by centrifugation at 12000 x g. Supernatants were subjected to X-ray fluorescence (XRF) analysis. Standards were prepared in
10 a matrix identical to that of the samples (i.e., normal tissues) to account for possible matrix interference. Administration of the complex (130 µg complexed Pt) resulted in liver accumulation of 70%/g tissue, i.e., about 90 µg Pt metal/gram liver (no figure), a value which falls within the
15 required range for contrast enhancement, as described below.

A mouse injected with such a preparation was subjected to X-ray mammography, along with a mouse which had been injected by the same amount of dextran solution with no Pt. As shown in Figs. 7A and 7B, the liver of the Pt-targeted
20 mouse is of a darker shade and is clearly delineated (Fig. 7A), whereas that of the control mouse is lighter and cannot be distinguished from other organs (Fig. 7B).

CDDP binds to CMdex in a reversible manner, i.e., it is released in biological systems in vivo in favor of
25 ligands exhibiting a higher affinity towards Pt. CDDP complexes of CMdex are toxic above a certain dose, with the toxicity being correlated with the dextran molecular weight.

Complexes of CMdex T-40 are similar to that of free CDDP in their activity, whereas complexes of higher dextrans are more
30 toxic.

The CDDP-CMdex-Hydr product (Protocol 2) prepared according to the present invention was non-toxic when tested by injecting the dialyzed complex at two doses 350 µg (13.5 mg/kg) and 630 µg (23.7 mg/kg) complexed CDDP. This is a
35 striking advantage over the use of CMdex-type polymer (T-250) that contains no hydrazide groups which is shown to have high toxicity of CMdex-CDDP complex (toxic already at 4 mg/kg)

(Schechter et al, 1989b).

Higher TNP and CDDP load can be achieved by forming a "dendrite" molecule of increased carboxyl groups content; CMdex reacted with iminodiacetic acid resulted in a molecule that could carry 150 mol TNP and up to 600 mol Pt/mol dextran-hydrazide, an advantageous product in view of its increased marker content and higher metal-load. Increasing the TNP ratio facilitates liver accumulation, whereas the high metal content enables the use of lower injected carrier doses.

In order to assess experimentally the minimal required dose of contrast agent and the feasibility of its selective delivery to the tissue, the contrast agent concentration level required for imaging was calculated from compiled X-raabsorption cross-section data and the sensitivity of standard X-ray detection techniques. To further assess these estimates, a Plexiglas phantom having various sized compartments and containing platinum chloride sample solutions of various concentrations was imaged by both CT (computed tomography) and standard film-based mammography.

An example of a CT scan through the phantom, showing the X-ray absorption by solutions of different Pt concentrations, is shown in Fig. 8. The results, summarized in Fig. 9, clearly support the theoretical estimate of about 100 µg/ml required for a detectable contrast change in CT. Similar results were obtained by film-based mammography.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the

principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

5 All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and
10 text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional
15 ~~method steps, known methods or conventional methods is not in~~
any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific
20 embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue
25 experimentation, without departing from the general concept of the present invention. Therefore, such adaptations modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be
30 understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with
35 the knowledge of one of ordinary skill in the art.

REFERENCES

- 5 Aboud-Pirak E, Hurwitz E, Bellot F, Schlessinger J, Sela M.
Inhibition of human tumor growth in nude mice by a conjugate
of doxorubicin with monoclonal antibodies to epidermal growth
factor receptor. Proc Natl Acad Sci USA 86:3778-3781, 1989.
- 10 Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by
targeted drug delivery to tumor vasculature in a mouse model.
Science 279:377-380, 1998.
- 15 Arnon R, Hurwitz E, Schechter B. The use of antibodies and
polymers as carriers of cytotoxic drugs in the treatment of
cancer. In: Horizons in Biochemistry and Biophysics. Vol. 9.
Palmieri, F. J. Wiley and Sons Ltd. (Eds), England. p.33-56,
1989.
- 20 Ashwell G, Harford J. Carbohydrate specific receptors in the
liver . Annu Rev Biochem 51:531-544, 1982.
- Bernstein A, Hurwitz E, Maron R, Arnon R, Sela M, Wilchek M.
Antitumor efficacy of daunomycin when linked to dextran: In
vivo and in vitro studies. Natl Cancer Inst 60:379-384, 1987.
- 25 Bevilacqua MP, Stengelin S, Gimbrowne MA, Jr, Seed B.
Endothelial leukocyte adhesion molecule 1: An inducible
receptor for neutrophils related to complement regulatory
proteins and lectins. Science 243:1160-1165, 1989.
- 30 Brooks PC, Montgomery AMP, Rosenfeld M, Reisfeld RA, Hu TH,
Klier G, Cheresh DA. Integrin alfa(V)beta(3) antagonists
promote tumor-regression by inducing apoptosis of angiogenic
blood-vessels. Cell 79:1157-1164, 1994.
- 35 Cepek KL, Shaw SL, Parker CM, et al. Adhesion between
epithelial cells and T lymphocytes mediated by E-cadherin and
the alpha E beta 7 integrin. Nature 372 :190-193, 1994.

- Dubois M, Gilles KA, Hamilton JK, Rebers Pa, Smith F.
Colorimetric method for determination of sugars and related
substances. Anal Chem 28:350-355, 1956.
- 5 Duncan R, Dimitrijevic S, Evagorou EG. The role of polymer
conjugates in the diagnosis and treatment of cancer. S. T.
P. Pharma Science 6:237-263, 1996.
- 10 Dunn RL and Ottenbrite RM (eds). Polymeric drugs and drug
delivery system. ACS Symposium Series 469, American Chemical
Society. Washington DC, 1991.
- 15 Epenetos AA, Snook D, Durbin H, et al. Limitation of
radiolabeled monoclonal antibodies for localization of human
neoplasms. Cancer Res. 4:3183-3191, 1986.
- Folkman J. Fighting cancer by attacking its blood supply. Sci
Am 275:116-119, 1996.
- 20 Fujita T, Nishikawa M, Ohtsubo Y, et al. Control of in vivo
fate of albumin derivatives utilizing combined chemical
modification. J. Drug Targeting 2: 157-165, 1994.
- 25 Golla ED, Ayers GH. Spectrophotometric determination of
platinum with O-phenylenediamine. Talanta 30:199-210, 1973.
- 30 Hashida M, Nishikawa M, Takakura Y. Hepatic targeting of
drugs and proteins by chemical modification. J Controlled
Release 36:99-107, 1995.
- Hoyle GW, Hill RL. Molecular cloning and sequencing of a cDNA
for a carbohydrate binding receptor unique to rat Kupffer
cells. J Biol Chem. 263:7487-7492, 1988.
- 35 Howe-Grant ME, Lippard SJ. Aqueous platinum (II) chemistry:
Binding to biological molecules, in: H. Sigel (Ed.) Metal
Ions in Biological Systems, Marcel Dekker, New York, NY
11:63-125, 1980.

Hunter WM, Greenwood FC. Preparation of iodine-131 labeled growth hormone of high specific activity. Nature (Lond) 194:495-496, 1962.

5

Hurwitz E, Wilchek M, Pitha J. Soluble macromolecules as carriers for daunorubicin. J Applied Biochem 2:25-35, 1980.

10

Johnson RC, Zhu D, Augustin-Voss HG, Pauli BU. Lung endothelial dipeptidyl peptidase IV is an adhesion molecule for lung-metastatic rat breast and prostate carcinoma cells. J Cell Biol 121:1423-1432, 1993.

15

Johnston GI, Cook RG, McEver RP. Cloning of GMP-140, a granulate membrane protein of platelets and endothelium: sequence similarity to proteins involved in cell adhesion and inflammation. Cell 56:1033-1044, 1989.

20

Kedar E, Rutkowski Y, Braun E, Emanuel N, Barenholz Y. Delivery of cytokines by liposomes. I. Preparation and characterization of interleukin-2 encapsulated in long-circulating sterically stabilized liposomes. J Immunother Emphasis Tumor Immunol 16:47-59, 1994.

25

Kikutani H, Inui S, Sata R, Barsumian EL, Owaki H, et al. Molecular structure of human lymphocyte receptor for immunoglobulin E. Cell 1986, 47: 657-665, 1986.

30

Larsen C. Dextran prodrugs - structure and stability in relation to therapeutic activity. Adv Drug Deliv Rev 3:103-154, 1989.

35

Lasky LA, Singer MS, Yednock TA, Dowbendo D et al. Cloning of a lymphocyte homing receptor reveals a lectin. Cell 56:1045-1055, 1989.

Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: mechanism of

tumoritropic accumulation of proteins and antitumor agent
Smancs. Cancer Research 46:6387-6392, 1986.

5. Monsigny M, Roche AC, Midoux P, Mayer R. Glycoconjugates as carriers for specific delivery of therapeutic drugs and genes. Adv Drug Deliv 14:1-24, 1994.

- 10 Neurath R, AK, Strick N, Li YY, Lin K, Jiang S. Blocking of CD4 cell receptors for human immunodeficiency virus type 1 (HIV-1) by chemically modified bovine milk proteins: Potential for AIDS prophylaxis. J. Molec Recog 8:304-316, 1995.

- ~~15 Pasqualini R, Ruoslahti E. Organ targeting in vivo using phage display peptide libraries. Nature 380:364-366, 1996.~~

- 20 Pimm MV, Perkins AC, Duncan R, Ulbrich K. Targeting of (2-hydroxypropyl)methacrylamide copolymer-doxorubicin conjugate to the hepatocyte galactose-receptor in mice: visualization and quantification by gamma scintigraphy - a basis for clinical targeting studies. J Drug Target 1:125-133, 1993.

- ~~25 Pimm MV, Perkins AC, Strohalm J, Ulbrich K, Duncan R. Gamma scintigraphy of the biodistribution of ¹²³I-labeled N-(2-hydroxypropyl)methacrylamide copolymer-doxorubicin conjugates in mice with transplanted melanoma and mammary carcinoma. J Drug Target 3:375-383, 1996.~~

- 30 Pokutta S, Herrenknecht K, Kemler R, Engel J. Conformational changes of the recombinant extracellular domain of E-cadherin upon calcium binding. Eur J Biochem 223:1019-1026, 1994.

- 35 Schechter B, Arnon R, Freedman Y E, Chen L, Wilchek M. Liver accumulation of TNP-modified streptavidin and avidin: Potential use for targeted radio- and chemotherapy. J Drug Targeting 4:171-179, 1996.

Schechter B, Pauzner R, Wilchek M, Arnon R.
Cis-platinum(II) complexes of carboxymethyl-dextran as
potential anti-tumor agents. I. Preparation and
characterization. Cancer Biochem Biophys 8:277-288, 1986.

5

Schechter B, Pauzner R, Arnon R and Wilchek M.
Cis-platinum(II) complexes of carboxymethyl-dextran as
potential anti-tumor agents. II. In vitro and in vivo
activity. Cancer Biochem Biophys 8:289-298, 1986.

10

Schechter B, Wilchek M, Arnon R. Increased therapeutic
efficacy of cis-platinum complexes of poly-L-glutamic acid
against a murine carcinoma. Int J Cancer 39:409-413, 1987.

15

~~Schechter B, Pauzner R, Wilchek M, Arnon R, Haimovich J.~~
Selective cytotoxicity against tumor cells by
cis-platin(II) complexed antitumor antibodies via
carboxymethyl dextran. Cance Immunol Immunother
25:225-, 1987.

20

Schechter B, Rosing MA, Wilchek M, Arnon R. Blood levels and
serum protein binding of cis-platinum (II) complexed to
carboxymethyl-dextran. Cancer Chemother Pharmacol
24:161-166 , 1989.

25

Schechter B, Neumann A, Wilchek, M, Arnon R. Soluble
polymers as carriers of cis-platinum. J Controlled Release
10:75-78 , 1989.

30

Schechter B, Arnon R, Wilchek M, Schlessinger J, Hurwitz E,
Aboud-Pirak E, Sela M. Indirect immunotargeting of cis-Pt to
human epidermoid carcinoma KB using the avidin-biotin
system. Int J Cancer 48:167-172, 1991.

35

Schechter B, Arnon R, Wilchek M. Polymers in drug delivery:
immunotargeting of carrier-supported cis-platinum (CDDP)
complexes. Reactive Polymers 25:167-175, 1995.

Spiess M, Schwartz AL, Lodish HF. Sequence of human asialoglycoprotein receptor cDNA. J Biol Chem. 1985; 260:1979-1982.

- 5 Taylor ME, Bezouska K, Drickamer K. Contribution to ligand binding by multiple carbohydrate-recognition domain in the macrophage mannose receptore. J Biol Chem. 267:1719-1726, 1992.

- 10 Varner JA, Cheresh DA. Tumor angiogenesis and the role of vascular cell integrin alphavbeta3. Important Adv Oncol 1996:69-87.

Warshawsky A, Fieberg MMB, Mihalik P, Murphy TG, Ras YB.

- ~~15 The separation of platinum group metals (PGM) in chloride media by isothiuronium resins. Separation & Purification Methods 9:209-265, 1980.~~

Warshawsky A. Selective ion exchange polymers. Angew.

- 20 Makromol Chem 109-110, 171, 1982.

Warshawsky A. Hydrometallurgical processes for the separation of platinum group metals in chloride media. In: Ion Exchange Technology (D. Naden and M. Streat, eds.) Ellis Horwood Publishers, pp.604-611, 1984.

25

Warshawsky A. Modern research in Ion exchange. In Science and Technology. A.E. Rodrigues (ed.), Martinus Nijhoff Publishers, Dordrecht, The Netherlands, pp.67-119 1986.

30

Warshawsky A. The extraction of platinum group metals by ion exchange. Ion Exchange and Sorbtion Processes in Hydrometallurgy. John Wiley and Sons, Chichester, England, Chap. 3. pp.127-165 1987a.

35

Warshawsky A. Chelating ion exchangers. Ion Exchange and Sorbtion Processes in Hydrometallurgy. John Wiley and Sons, Chichester, England, Chap. 4. pp.166-226 1987b.

Warshawsky A. Polymeric ligands in hydrometallurgy. Syntheses and separations using functional polymers. D.C. Sherrington and P. Hodge (eds.) John Wiley and Sons, Chichester, England, Chap. 14. pp.325-385 1987.

Welch DR. Biologic considerations for drug targeting in cancer patients. Cancer Treat Rev 14:351- 358, 1987.

10

WHAT IS CLAIMED IS:

1. A delivery system for targeting a contrast agent to specific organs for the purpose of tumor diagnosis, prognosticating the effectiveness of chemotherapy in the treatment of cancer and for follow-up of cancer therapy by X-ray imaging, wherein said delivery system comprises a conjugate or complex of a macromolecular carrier and a contrast agent selected from the group consisting of: (a) a compound having a heavy element with an atomic number in the range of 75 to 92, and (b) a compound having an element with a property of an abrupt change in its X-ray attenuation coefficient within the energy range used for radiography, said macromolecular carrier of said conjugate or complex being optionally linked to a specific tissue marker molecule.

2. The delivery system according to claim 1, wherein said contrast agent is a heavy metal compound.

3. The delivery system according to claim 2, wherein said heavy metal is selected from the group consisting of Pt, Au and Tl.

4. The delivery system according to any one of claims 1 to 3, wherein the macromolecular carrier is selected from the group consisting of liposomes, natural polymers and synthetic polymers.

5. The delivery system according to claim 4, wherein said macromolecular carrier is a natural or synthetic polymer to which said heavy metal compound is attached through a metal-binding group or ligand containing nitrogen, nitrogen-oxygen or sulfur atoms.

6. The delivery system according to claim 4, wherein said macromolecular carrier is a natural or synthetic polymer to which said heavy metal compound is complexed through a metal-chelating ligand.

7. The delivery system according to claim 6, wherein said metal-chelating ligand is selected from the group consisting of iminodiacetic acid, EDTA, DTPA, hydroxyquinoline, hydroxamic acid, hydrazide and combinations thereof.

8. The delivery system according to any one of claims 3 to 7, wherein said complex of a macromolecular carrier and a contrast agent is a Pt compound-dextran-hydrazide complex.

5 9. The delivery system according to claim 1, wherein said element in (b) has a property of an abrupt change in its X-ray attenuation coefficient within the energy range of 10 to 30 keV.

10 10. The delivery system according to claim 8, wherein said element having a property of an abrupt change in its X-ray attenuation coefficient is an element with an atomic number in a range of 33 to 50.

11. The delivery system according to claim 9 or 10, wherein said element is Zr.

15 12. The delivery system according to claim 1, wherein said element in (b) has a property of an abrupt change in its X-ray attenuation coefficient at an energy range above 30 keV and has an atomic number above 50.

20 13. The delivery system according to any one of claims 1 to 12, wherein said macromolecular carrier in said conjugate or complex is linked to a specific tissue marker.

25 14. Use of a delivery system for the preparation of a composition useful for targeting a contrast agent to specific organs for the purpose of tumor diagnosis, prognosticating the effectiveness of chemotherapy in the treatment of cancer and for follow-up of cancer therapy by X-ray imaging, comprising a conjugate or complex of a carrier, and a contrast agent selected from the group consisting of a compound selected from the group consisting of: (a) a compound having a heavy element with an atomic number in a range of 75 to 92, and (b) a compound having an element having a property of an abrupt change in its X-ray attenuation coefficient within the energy range used for radiography, where said macromolecular carrier is optionally
30 linked to a specific tissue marker.

35 15. A method for tumor diagnosis, comprising the steps of:

administering to a patient an effective amount of a

delivery system according to any one of claims 1 to 13; and
X-ray imaging the patient to diagnose the presence
of a tumor.

5 16. A method for prognosticating the effectiveness
of chemotherapy in the treatment of cancer, comprising the
steps of:

administering to a patient an effective amount of a
delivery system according to any one of claims 1 to 13; and

10 X-ray imaging the patient to prognosticate the
effectiveness of chemotherapy in treating cancer.

17. A method for follow-up of cancer therapy by
X-ray imaging, comprising the steps of:

administering to a patient an effective amount of a
delivery system according to any one of claims 1 to 13; and

~~15 X-ray imaging the patient to determine the
effectiveness of cancer therapy.~~

18. A method according to any one of claims 15-17
for diagnosis, prognosis or follow-up of therapy of breast
cancer.

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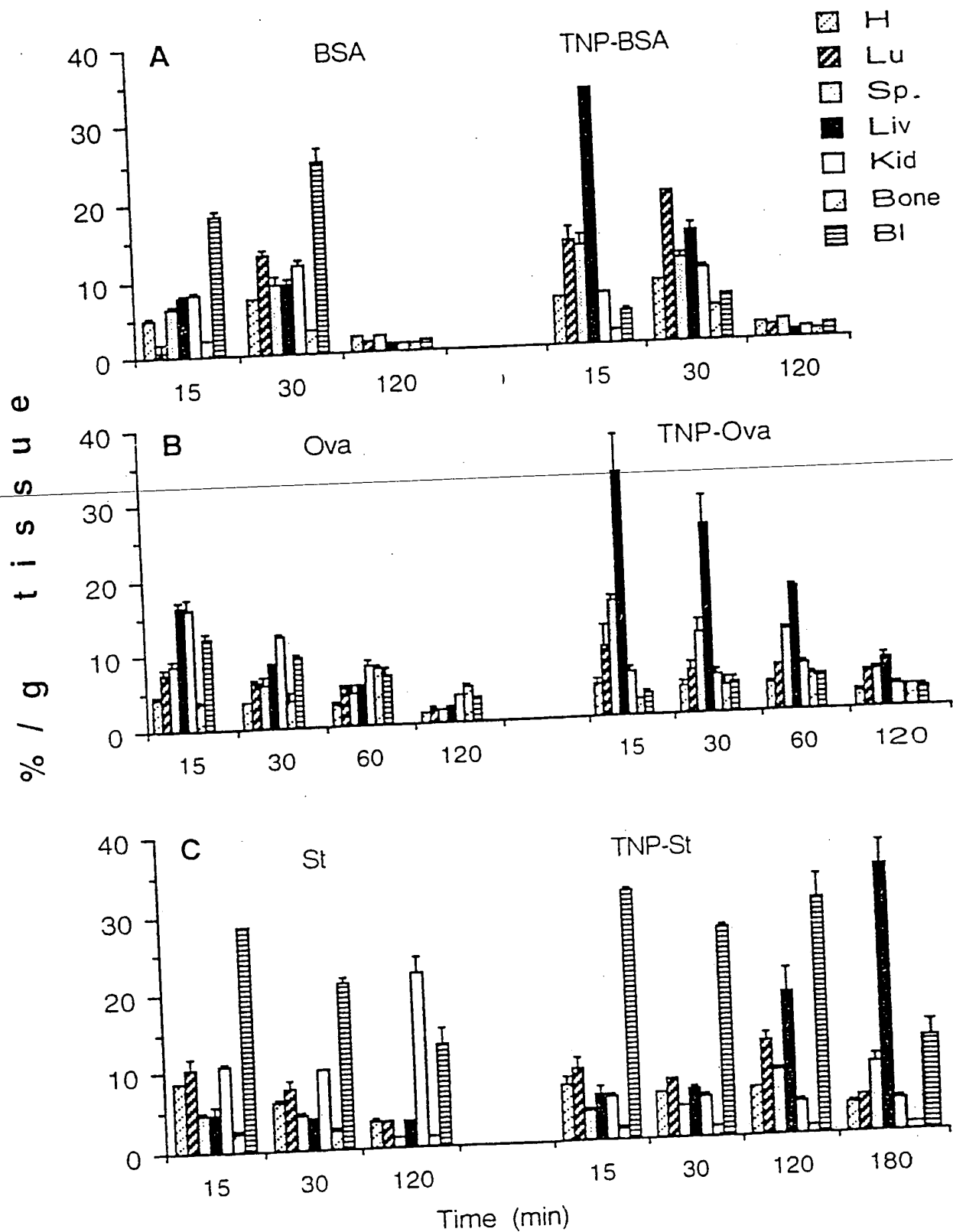


Figure 1

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Figure 2

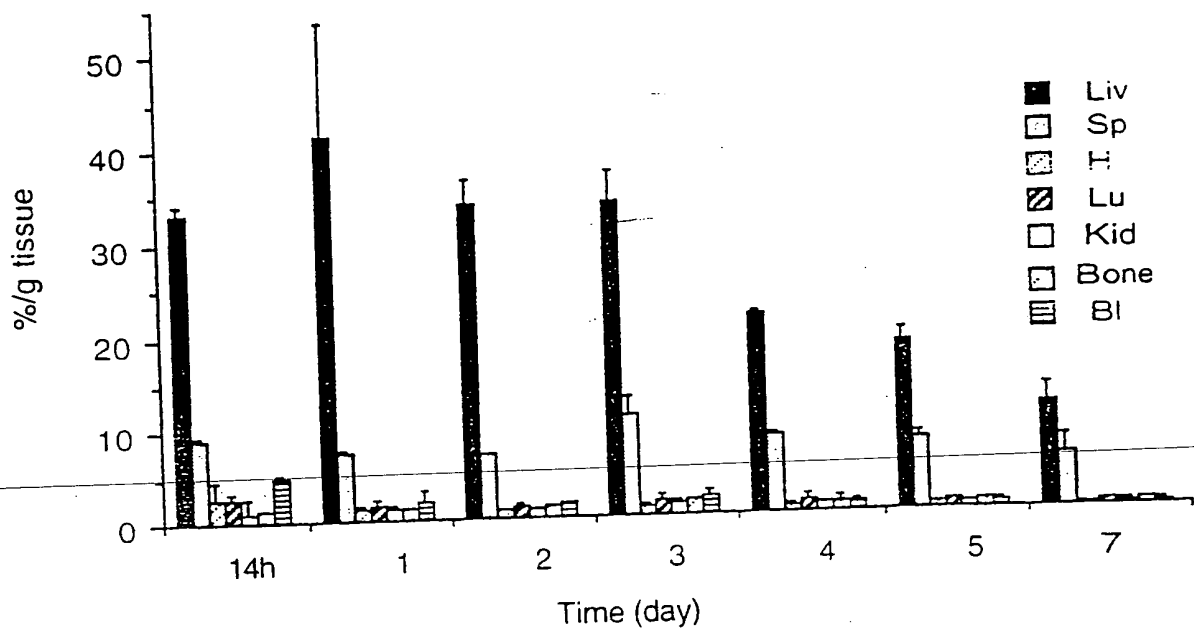
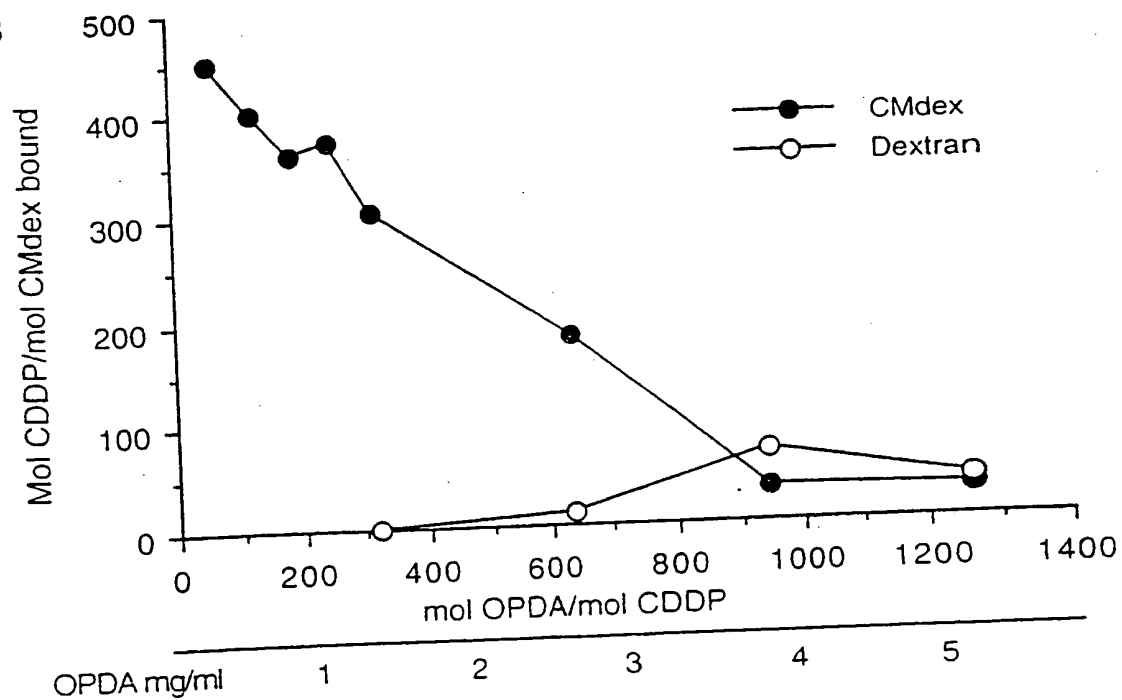
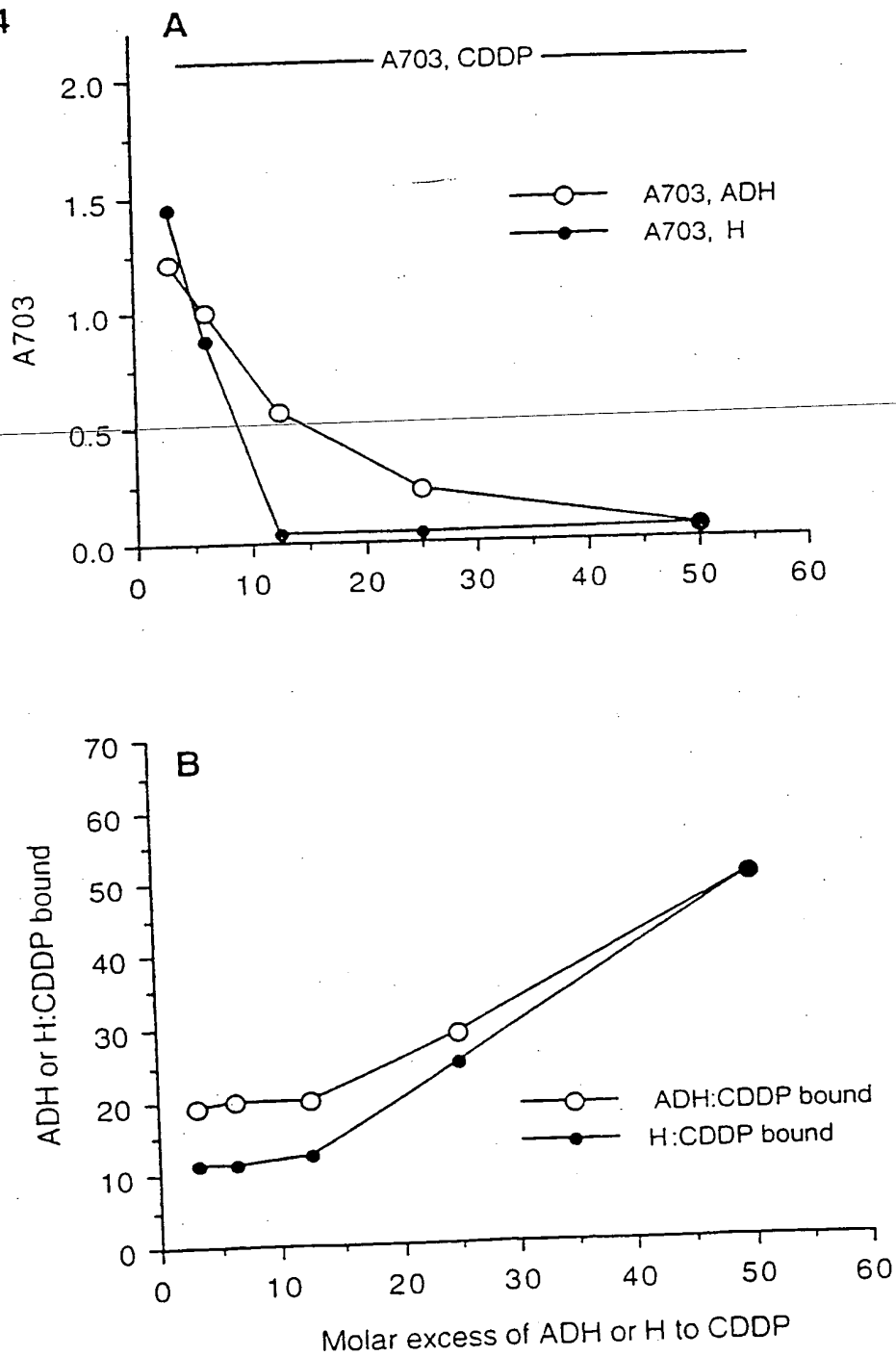


Figure 3



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Figure 4



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Figure 5

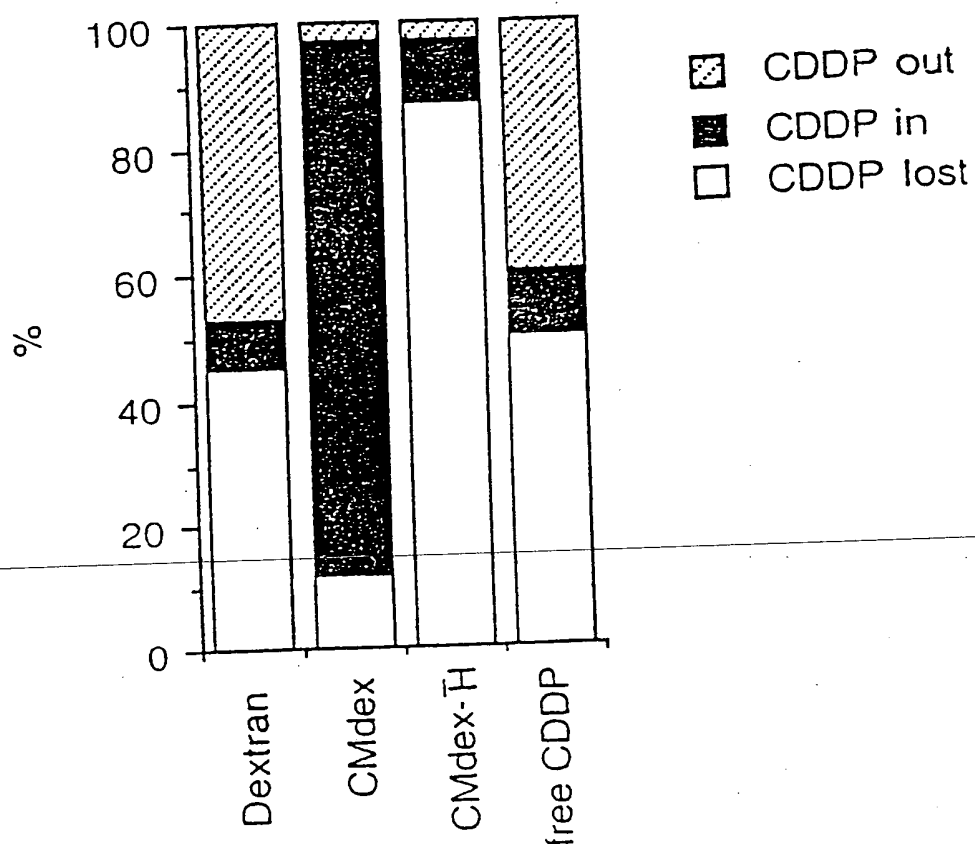
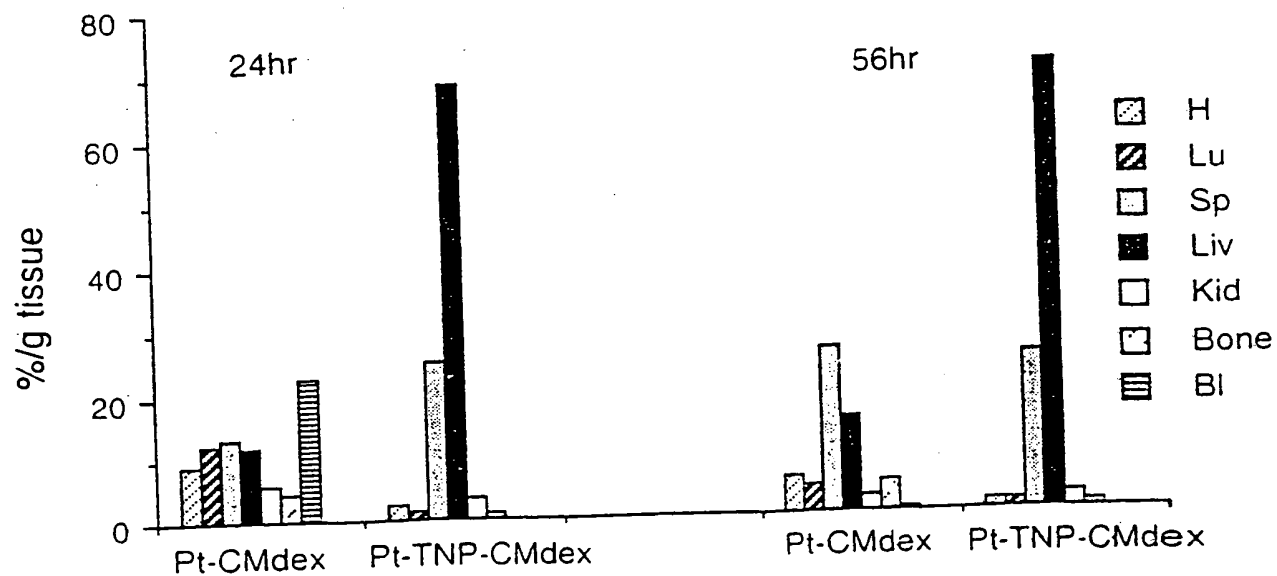


Figure 6



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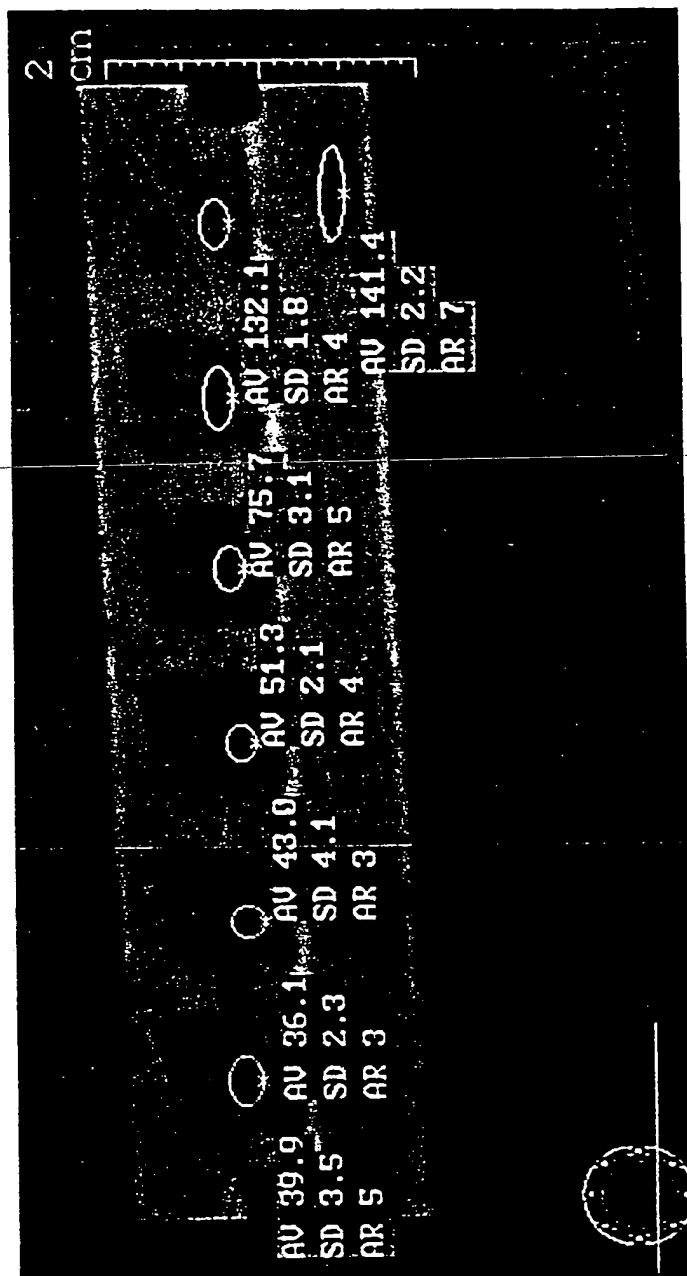


Figure 7

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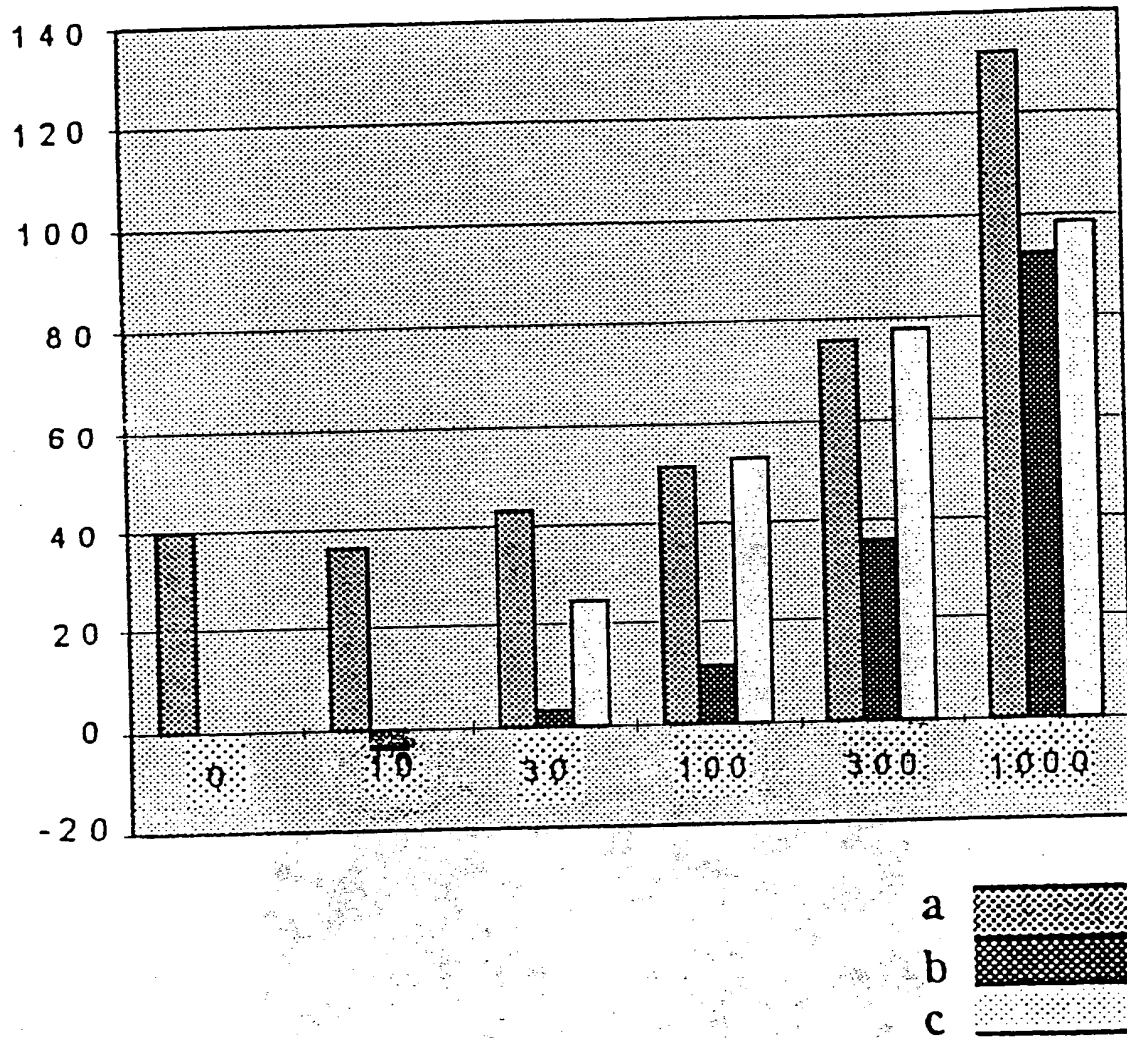


Figure 8

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Fig. 9

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IL 98/00282

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K49/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 190 464 A (YEDA RES & DEV) 13 August 1986 see page 5, paragraph 2 see page 6, paragraph 1: claims 1,6,9,10,14; example 2 ---	1-18
A	KNEBEL, NORBERT G. ET AL: "2-Phenylindole-linked (2-(aminoalkyl)pyridine)dichloroplatinum(I I): complexes with a selective action on estrogen receptor positive mammary tumors" J. MED. CHEM., 1991, 34, 2145-52. XP002083595 see abstract see page 2148, paragraph 4: table 5 --- -/--	1-18



Further documents are listed in the continuation of box C



Patent family members are listed in annex

Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
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- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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- "&" document member of the same patent family

Date of the actual completion of the international search

9 November 1998

Date of mailing of the international search report

27/11/1998

Name and mailing address of the ISA

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Authorized officer

Gonzalez Ramon, N

INTERNATIONAL SEARCH REPORT

International application No

PCT/IL 98/ 00282

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 15-18
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 15-18
are directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

In. tional Application No
PCT/IL 98/00282

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 94 17829 A (NEORX CORP) 18 August 1994 see page 15, line 27-30 see page 17, line 29 see figure page 27 see page 19, line 15-30; claims 2.11.12; examples 2-5,12.13 ---	1-7, 12-17
X	WO 94 23759 A (IMMUNOMEDICS INC) 27 October 1994 see page 25, line 28-39; claims 12,27,29,42,44; examples 10,11 see page 23, line 25 - page 24, line 25 ---	1-7, 12-17
X	WO 94 02068 A (GEN HOSPITAL CORP) 3 February 1994 see claims 12,21,32,42; examples 10,15,20 see examples 2,5,6 ---	1-7, 12-18
X	WO 96 00079 A (GEN HOSPITAL CORP ;BOGDANOV ALEXEI (US); BRADY THOMAS J (US); WEIS) 4 January 1996 see abstract; claims 1,3,21,23,25,30; figures 2A,2B; example 15 ---	1-7, 12-18
X,P	WO 97 22879 A (ARNON RUTH ;SCHECHTER BILHA (IL); WILCHEK MEIR (IL); YEDA RES & DE) 26 June 1997 see page 13, line 25-35; example 8 see page 37, line 6-16; claims 19-21 ---	1-7, 12-17
X	SCHECHTER B ET AL: "POLYMERS IN DRUG DELIVERY: IMMUNOTARGETING OF CARRIER-SUPPORTED CIS-PLATINUM COMPLEXES" REACTIVE POLYMERS, vol. 25, no. 2/03, June 1995, pages 167-175, XP000647654 see abstract ---	1-7, 12-17
X,P	AVICHEZER D ET AL: "Functional polymers in drug delivery: carrier-supported CDDP (cis -platin) complexes of polycarboxylates -- effect on human ovarian carcinoma" REACTIVE & FUNCTIONAL POLYMERS, vol. 36, no. 1, February 1998, page 59-69 XP004111446 see discussion see abstract -----	1-7, 12-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IL 98/00282

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0190464	A	13-08-1986	AU 5173685 A	10-07-1986
			JP 61222927 A	03-10-1986
WO 9417829	A	18-08-1994	CA 2154667 A	18-08-1994
			EP 0683676 A	29-11-1995
			US 5607659 A	04-03-1997
WO 9423759	A	27-10-1994	US 5482698 A	09-01-1996
			AU 674580 B	02-01-1997
			AU 6666694 A	08-11-1994
			CA 2161109 A	27-10-1994
			EP 0695194 A	07-02-1996
			JP 8509226 T	01-10-1996
			SG 46472 A	20-02-1998
WO 9402068	A	03-02-1994	AU 4778893 A	14-02-1994
			EP 0654973 A	31-05-1995
			JP 7509467 T	19-10-1995
			US 5582172 A	10-12-1996
WO 9600079	A	04-01-1996	AU 2701395 A	19-01-1996
WO 9722879	A	26-06-1997	AU 1686397 A	14-07-1997

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